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Gene Regulation Mediated by Interaction between HTLV-1 Promoter Elements and Transcription Factors Tax and CREB

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In this work we examine the role of three genetic control components in the regulation of HTLV-1 transcription: cyclic AMP-responsive element (CRE)-binding protein (CREB), the HTLV-1 trans-activator Tax, and the three Tax-responsive elements (TREs). We demonstrate that the *in vivo* efficiency of the HTLV-1 promoter basal expression in cell culture depends on the spacing between the three TRE elements, located at the HTLV-1 LTR (long terminal repeat), whereas the level of transcription activation mediated by Tax is affected by the number of TREs. In the presence of only one TRE, the enhancement of expression by Tax is affected by the distance between the single TRE and the transcription start site. Following CREB binding to the LTR, additional DNase I hypersensitive sites are generated in the region between the two distal TREs (I and II), while in the presence of Tax, such sites are generated also in the region between TREs II and III. Neither cooperative binding of CREB to the TREs nor preferential binding of CREB to a particular TRE was observed. Tax binding to the CREB/TRE complex does not change the DNase I protection pattern. Taken together, these results suggest that the basal CREB-mediated transcription is determined by the number and the position of the viral TREs relative to each other. Tax protein stabilizes the protein/DNA complex and suppresses the spacing limitations, probably by bridging between the CREB/TRE complexes and the basal initiation transcription complex.

INTRODUCTION

The retrovirus human T-cell lymphotrophic virus type 1 (HTLV-1) has been implicated in an aggressive lymphoproliferative disorder termed adult T-cell leukemia (ATL) and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain *et al.,* 1985; Poiesz *et al.,* 1980; Seiki *et al.,* 1982; Yoshida *et al.,* 1982). The viral genome encodes a unique oncoprotein called Tax. This protein is a potent transcription transactivator that regulates HTLV-1 replication and facilitates the transition from quiescent infection to high levels of virus production in T-cells (Felber *et al.,* 1985; Slamon *et al.,* 1984; Sodroski *et al.,* 1985). The *tax* gene product was also found to affect the expression of a variety of cellular and viral genes (Bohnlein *et al.,* 1988; Franklin and Nyborg, 1995; Fujii *et al.,* 1992; Shannon *et al.,* 1995; Suzuki *et al.,* 1993a). Similar to the herpes simplex virus VP16 and the adenovirus E1a viral transactivators, Tax does not bind directly to DNA or RNA but rather activates transcription by modulation of various host transcription factors (Armstrong *et al.,* 1993; Fujii *et al.,* 1992, 1994; Uittenbogaard *et al.,* 1994; Yoshida, 1996).

A variety of cellular factors interact with the HTLV-1 LTR (long terminal repeat), such as the members of the CREB/ATF family, TIF, THP, HEBI, TFIID, SPI, and others (Beraud *et al.,* 1991; Bosselut *et al.,* 1982, 1990; Duvall *et al.,* 1995; Jeang *et al.,* 1988; Marriott *et al.,* 1990; Nybrog and Dynan, 1990; Muchardt *et al.,* 1992; Tanimura *et al.,* 1993; Yin and Gaynor, 1996; Yoshimura *et al.,* 1990; Zhao and Giam, 1991). Tax interacts with the HTLV-1 LTR indirectly via binding to the nuclear factor CREB, which binds to three incomplete CRE sites located within the core of unique 21-bp repeats, termed TRE (Beimling and Moelling, 1992; Brauweiler *et al.,* 1995; Fujisawa *et al.,* 1989; Jeang *et al.,* 1988; Shimotohno *et al.,* 1986; Suzuki *et al.,* 1993b). The G/C-rich sequences immediately flanking the CRE motif in the 21-bp repeat are crucial for the formation of stable Tax/CREB/DNA ternary complexes *in vitro* (Anderson and Dynan, 1994; Paca-Uccaralertkun *et al.,* 1994) and for Tax-mediated transactivation *in vivo* (Fujisawa *et al.,* 1989). Recent studies demonstrated that Tax contacts the DNA within the G/C-rich region (Kimzey and Dynan, 1998; Lenzmeier *et al.,* 1998). The contact is at symmetric positions 14 nucleotides on either side of the CRE site, within the G/C-rich region (Kimzey and Dynan, 1998). It was shown that Tax stabilizes CREB/DNA complexes and enhances the binding affinity of CREB to the viral 21-bp repeat (Brauweiler *et al.,* 1995; Yin and Gaynor, 1996; Zhao and Giam, 1992). Biochemical and genetic experiments have demonstrated that the Tax domain responsible for the interaction with CREB maps to the amino-terminal cysteine-rich region, whereas the

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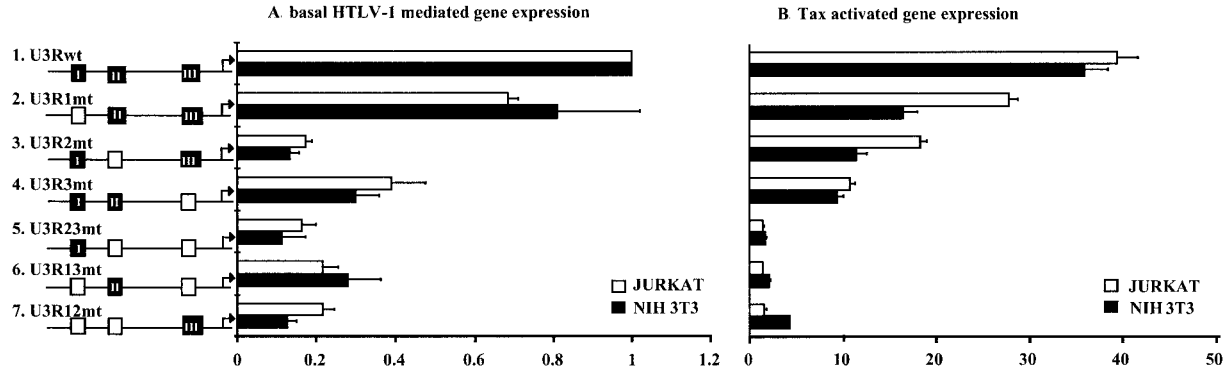

FIG. 1. Mutational analysis of the effect of the TRE sequences on HTLV-1-mediated gene expression. A schematic illustration of the promoter region of HTLV-1 fused to the firefly *luc* gene is presented in the left part of the figure. The black boxes represent the wild-type TREs and the open boxes the CRE-mutated TREs. Roman numbers identify the TRE on the HTLV-1 promoter. The transcription start site is indicated by the broken arrow. NIH3T3 and Jurkat cells were transfected with one of the plasmids listed (A) or cotransfected with pCTax, a Tax-producing plasmid (B). As a control for transfection efficiency, plasmid pRLU3R or pRL-CMV, in which the *luc* gene was replaced with the Renilla *luc* gene, was included in each transfection. Luc activity was normalized to that of the Renilla Luc activity. The bars represent Luc activity of each construct relative to basal activity of pU3Rwt, which was set to 1. The standard deviation for each plasmid construct activity is indicated.

carboxyl-terminal domain was implicated in the interaction of Tax with the basal transcription machinery (Adya and Giam, 1995; Goren *et al.,* 1995; Seiki *et al.,* 1982; Yin *et al.,* 1995).

The CREB protein is phosphorylated by protein kinase A as a response to cAMP signaling. Only phosphorylated CREB bound to CRE sites activates transcription via CREB binding protein (CBP) (Chrivia *et al.,* 1993). However, in the presence of Tax, CBP can be recruited to the viral TRE–CREB complex in a phosphorylation-independent manner, thereby serving as a cofactor for Tax activation of HTLV-1 transcription (Giebler *et al.,* 1997; Kwok *et al.,* 1996).

Although the mode of interaction between CREB, Tax, and the TREs has been extensively studied, the molecular mechanism and the role of each element of the triple complex in the regulation of HTLV-1 expression are not yet clear. Most studies investigated the CREB or CREB/Tax interaction with a single TRE in model systems. To elucidate the mode of activation of HTLV-1 transcription replication further, we investigated, *in vitro* and *in vivo,* in cultured cells, the role of each TRE and the possible interaction between them, as well as the effect of the TRE intervening sequences, in the CREB and the CREB/Tax-mediated gene expression in the HTLV-1 base composition.

RESULTS

Mutational analysis of the TREs in basal and Tax-mediated gene expression

Gene expression mediated by HTLV-1 LTR can be formally divided into two levels of expression: the CREB-dependent basal level and the viral Tax-activated level. Both levels of transcription activation depend upon interaction of the transcription factor with the HTLV-1 *cis*-acting TRE repeats.

To elucidate the contribution of each TRE site to HTLV-1 promoter expression and the possible interaction between them, we constructed expression vectors based on transcription fusion between HTLV-1 LTR and the firefly luciferase (*luc*) reporter gene. Mutations abolishing CREB binding were introduced in the various CRE-like sites, generating expression plasmids harboring mutations in either one of the TREs separately or plasmids harboring any combination of two mutated TREs (see Fig. 1). Luciferase expression was determined (as described under Materials and Methods) in NIH 3T3, Jurkat (Fig. 1), and 293 cell lines, in the absence of Tax (promoter basal expression) or following cotransfection with Tax expressing vector pCTax (Factor and Shaul, 1990). As an internal control for transfection efficiency, light emission derived from cotransfected plasmid pRLU3R in which the Renilla luciferase was expressed under the control of the HTLV-1 LTR or pRL-CMV was determined. All *luc* measurements were normalized to the amount of the gene expression of the control plasmids. It should be noted that cotransfection with either one of the latter plasmids did not affect the expression of the *luc* gene from the reporter plasmids. The use of two different *luc* genes as reporters, using the same measuring technique, leads to a more reliable normalization than using two reporter systems that rely on different protocols of lysis and measurement of the gene products. Each experiment was repeated at least three times (see standard deviation, Fig. 1).

The most prominent effect on basal HTLV-1 LTR-mediated gene expression resulted from abolishing CREB binding to the second 21-bp repeat (U3R2mt, Fig. 1). A mutation in the CRE sequences of TRE II that prevents binding of CREB to this site (cf. Fig. 2, lines 2–5 to lines 7–10) resulted in about 90% reduction in the basal CREB-dependent promoter activity in all three cell lines (NIH

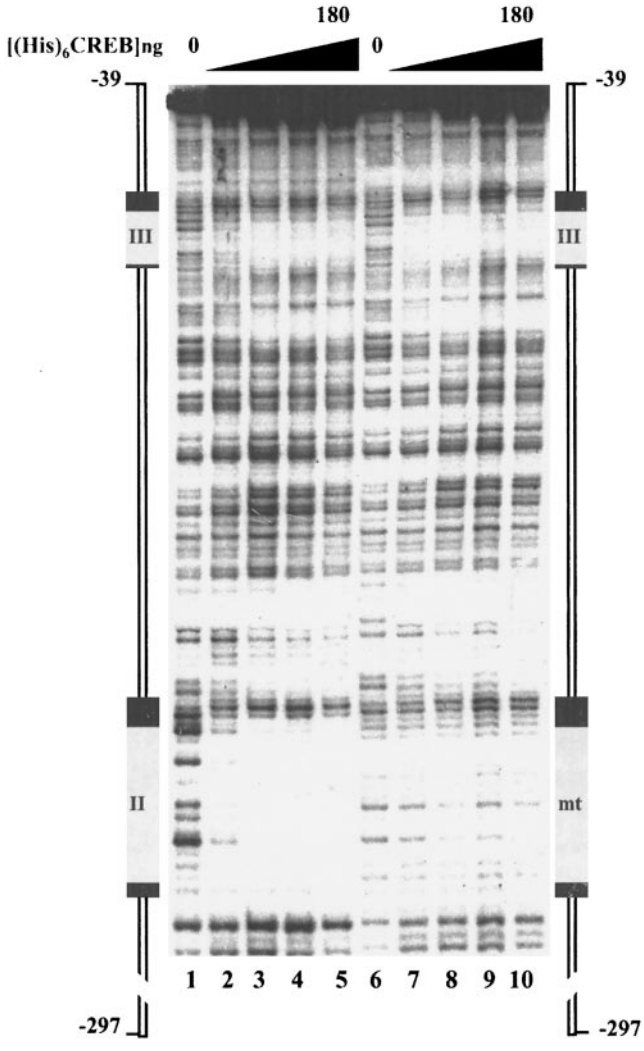

FIG. 2. DNase I protection analysis of CREB binding to the HTLV-1 LTR. HTLV-1 DNA probes (nucleotides −297 to −39, relative to the transcription start site) were subjected to DNase I digestion following preincubation with increasing amounts of $(His)_6$CREB protein (0, 45, 90, 120, and 180 ng). DNA digestion products (sense strand) of wild-type HTLV-1 LTR (lanes 1–5) or TRE II mutated LTR (lanes 6–10) were analyzed on sequencing gels. A schematic diagram of the wild-type and the TRE II-mutated LTR is presented on the left and the right sides of the figure, respectively. The TREs are specified by boxes, the CRE-mutated TRE II (mt) is indicated by an empty box, the TREs sequences protected by CREB are indicated by the light part of the boxes, and the unprotected part of the TREs is indicated by the black parts of the boxes.

3T3 and Jurkat cells are presented in Fig. 1). Promoter activity mediated by this site alone supports three times higher basal expression of the HTLV-1 LTR (U3R13mt) than either TRE I or TRE III alone or the combined activity of the two TRE sites together (Fig. 1). These results are in general agreement with the recent work of Barnhart *et al.* (1997) carried out in the HeLa cell line. Our results differ from those of Barnhart *et al.* (1997) as to the effect of the mutations in TRE I and TRE III on gene expression. In our work the mutation in TRE I only slightly affected the basal promoter activity, while mutating TRE III reduced the basal HTLV-1 LTR-mediated gene expression by about threefold (Fig. 1). In brief, one can conclude that TRE II plays a major role in the CREB-mediated HTLV-1 promoter activity.

Unlike the basal expression level, Tax-activated gene expression was determined mainly by the number of TRE sites rather than by a particular TRE (Fig. 1, cf. lines 2–4 to 5–7). Tax almost compensated for the absence of one TRE, resulting in similar levels of *luc* gene expression, 40 to 50% relative to the wild-type LTR in any of the three cell lines (Fig. 1B for NIH3T3 and Jurkat). On the other hand, one TRE supported only 3–13% of gene expression relative to that of wild-type promoter (Fig. 1B, lines 5–7). TRE III, the closest to the TATA box, responds best to Tax-mediated activation (Fig. 1B, lane 7). Thus, while a mutation in TRE II (U3R2mt) resulted in about 90% reduction in *luc* basal line expression, in the presence of Tax, *luc* activity was increased 130-fold, more than twice the fold of activation determined for the wild-type promoter (Fig. 1, lines 1 and 3).

To test whether this result is due to the minor difference between the sequences of TRE II and I or due to the relative distance from TRE II to the downstream TRE III, TRE II was moved 48 bases to the position of TRE I in the plasmid pGLU3R1mt. To reposition TRE II, an *Eco*RI site was generated 3′ to the TRE II (GA to TT substitution at position −180). This mutated LTR plasmid, pGLU3R1mt*E* (see Fig. 3), did not exhibit any change in gene expression relative to the parental vector pGLU3R1mt (cf. Fig. 3, lines 3 to 2). A PCR duplication of the 48 bases of the sequence located between TRE II and TRE III (−138 to −183 and an additional 2 bases) or a 48-base-long oligonucleotide of an arbitrary sequence was inserted at the *Eco*RI site, generating the expression plasmids pGLU3R1mt21d and pGLU3R1mt210, respectively (Fig. 3, lines 4 and 5). The CREB basal activity and Tax activation of gene expression of these two plasmid constructs were similar to those of U3R2mt, which harbors a mutated TRE II, in either NIH 3T3 or Jurkat (cf. Fig. 3, lines 4 and 5 to line 6).

In summary, the contribution of each TRE to the basal level of gene expression is different and depends on its location relative to the transcription start site. Tax activation levels, on the other hand, are affected by the number of TREs rather than by their relative position.

Interactions between CREB or CREB/Tax and the promoter region of HTLV-1

In HTLV-1 LTR the three 21-bp repeats are arranged in tandem and separated by 27 and 79 bp (Fig. 4B). Comparative studies of CREB binding to double-strand DNA oligonucleotides, corresponding to either one of the 21-bp repeats, indicate no preferential binding of CREB (our unpublished results and Anderson and Dynan, 1994;

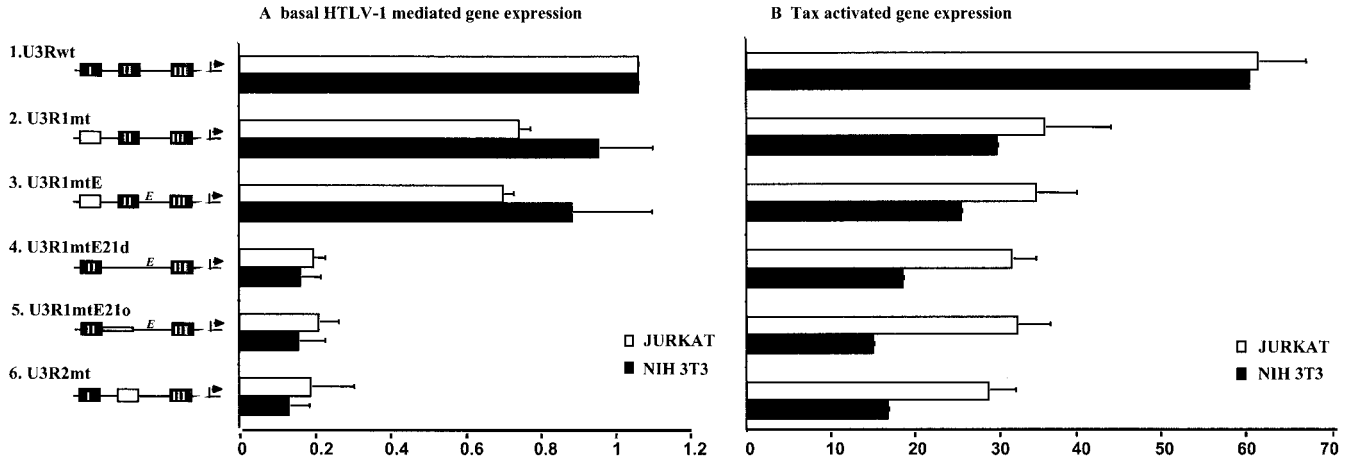

FIG. 3. Position effect of the TRE II on HTLV-1-mediated gene expression. A schematic illustration of the promoter region of HTLV-LTR, wild-type and mutants fused to the firefly *luc* gene is presented in the left part of the figure as described in the legend to Fig. 1. The *Eco*RI site is indicated by *E*. NIH3T3 cells were transfected with one of the plasmids listed (A) or cotransfected with pCTax (B). As a control for transfection efficiency, plasmid pRLU3R was added to each transfection. Results were calculated as described in the legend to Fig. 1.

Brauweiler *et al.,* 1995). Yet our observations demonstrate (see above) that mutating any one of TREs affects the basal and the Tax-mediated gene expression differentially. To further elucidate this finding and to verify that there is no preferential binding of CREB to individual TREs within the context of the complete LTR, we determined the DNase I protection patterns of CREB binding to the U3 portion of HTLV-1 LTR harboring all three TREs (nucleotides −39 to −297). These *in vitro* experiments, similar to the CREB binding experiments to individual 21-bp repeats, did not reveal any preferred CREB binding site. An increase of CREB concentration in those experiments gradually and equally enhanced the binding to all three TRE sites (see Fig. 4). Taken together with the results in the previous section, it may be suggested that the differential influence of each TRE on gene expression may result from the difference in the length of the TRE intervening sequences between the 21-bp repeats. This conclusion is supported by the finding that no significant difference in the binding of CREB, or CREB and Tax, to the 21-bp TRE sequence, flanked either by 45 bp of HTLV-1 sequence or by heterologous sequences when cloned in pBend 2, was observed in gel-shift experiments (EMSA) (not shown).

CREB protects three regions of the HTLV-1 LTR, spanning the nucleotide sequence −251 to −236, −203 to −188, and −103 to −88 on the sense strand, protecting the CRE core element and six bases 5′ and two bases 3′ of the core flanking sequences of each TRE (Figs. 4 and 5). The same protection pattern of the CRE core and flanking sequence on each TRE also emerged on the antisense strand (example of the protection of TRE II, Fig. 5B). Thus, the protected site is symmetrical and staggered. The first, second, and third protected TRE tracts are separated by about 32 and 84 bp, respectively (see Fig. 4B).

Using this DNase I protection assay, addition of Tax to the binding assay revealed only a faint change in the length of the protected region, (cf Fig. 5A, lanes 3–5 and lanes 7–9, to lanes 2 and 6, respectively; see also Paca-Uccaralertkun *et al.,* 1994). This change in the protected TRE sequence is much more obvious using the MPE:Fe footprinting analysis recently described by Lenzmeier *et al.* (1998). It is clear that in the presence of Tax, CREB protection of the TRE occurs at a lower concentration of CREB (cf. Fig. 5A, lane 5 to lane 6).

Analysis of the DNase I unprotected intervals between the TREs, in the presence of CREB, reveals DNase I hypersensitive sites between TRE I and II, but not between TRE II and III (Figs. 4 and 5, boldface arrowheads). The hypersensitive bands are not observed when the DNA fragment harbors only one TRE site (data not shown). These observations may suggest that CREB dimers cooperatively impose a conformational change of the DNA located between TRE I and TRE II.

Tax enhances only slightly the intensity of the DNase I hypersensitive bands between TRE I and II (Fig. 4, cf. lanes 2 and 6 to lanes 3–5 and 7–9). However, addition of Tax to the DNase I footprint assay led to the generation of hypersensitive bands in the unprotected interval between TRE II and TRE III (Figs. 5A and B, open arrows). It is thus possible that Tax changes the mode of interaction between these TREs (see Discussion).

DISCUSSION

We have directed our studies to elucidate the mode of interaction and function of the three transcription regulatory elements: the transacting cellular protein CREB, the viral transactivator HTLV-1 Tax, and the *cis*-acting HTLV-1 21-bp DNA repeats, the TREs. Although the mode of interaction between CREB, Tax, and the TREs has

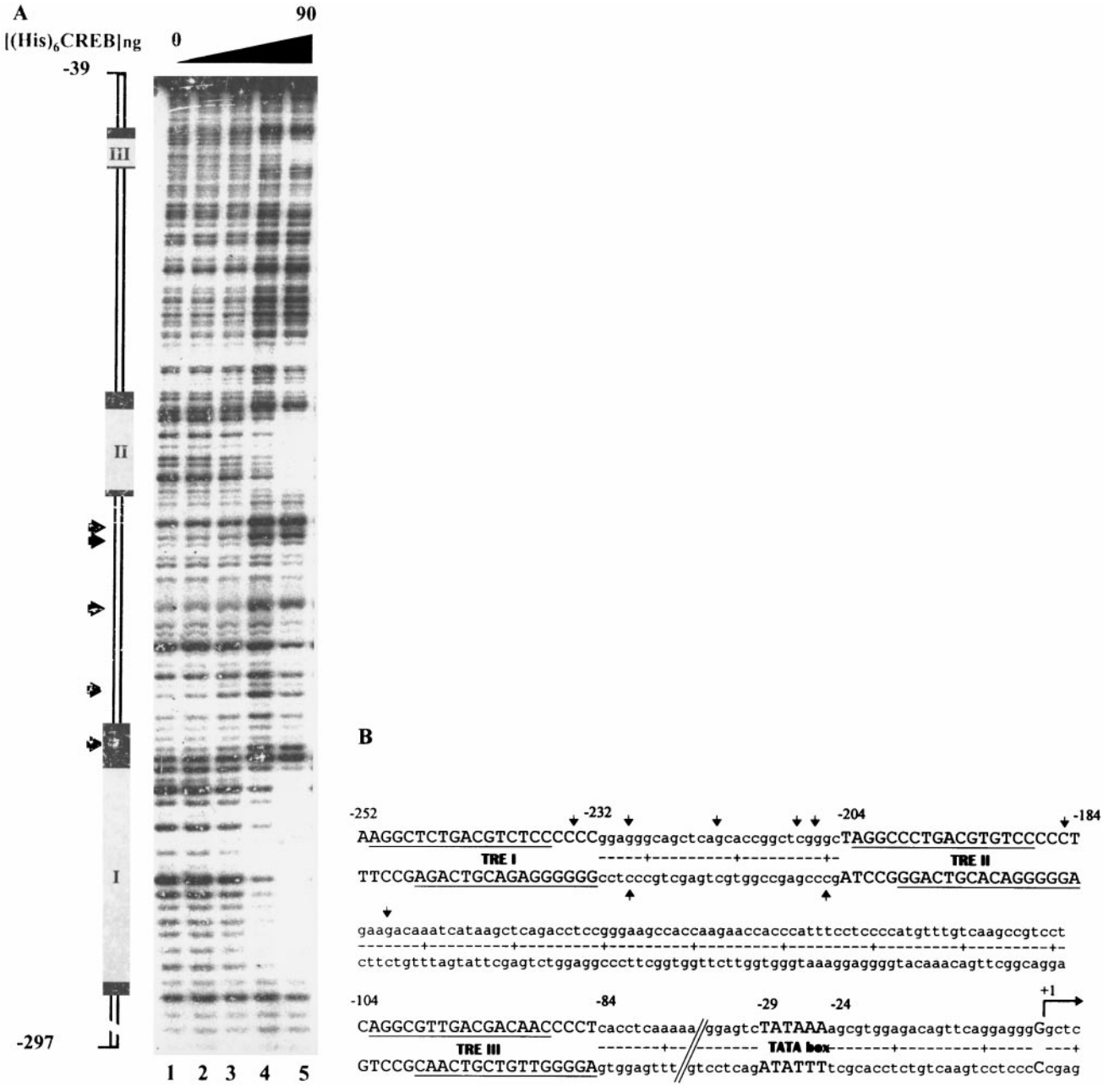

FIG. 4. DNase I protection analysis of CREB binding to the HTLV-1 LTR. HTLV-1 DNA probes (nucleotides −297 to −39, relative to the transcription start site) were subjected to DNase I digestion following preincubation with increasing amounts of $(His)_6$CREB protein (0, 15, 30, 45, and 90 ng). DNA digestion products of the HTLV-1 LTR were analyzed (sense strand) on sequencing gels. (A) Schematic diagram of the HTLV-1 LTR DNA probe is presented on the left side of the figure. The TREs are specified by boxes (see legend to Fig. 3). The DNase I hypersensitive bands between TRE I and TRE II are indicated by arrows. (B) DNA sequence of the relevant part of the HTLV-I LTR. The sequence of the CREB protected regions (underlined) of the TREs (boldface capital letters) and the hypersensitive DNase I sites (arrows) are indicated.

been extensively studied, the molecular mechanism and the role of each element of the triple complex in the regulation of HTLV-1 expression are not yet clear.

We studied this complex transcription control mechanism on two levels: the basal CREB-dependent and the CREB/Tax-activated gene expression. The contribution of the HTLV-1 LTR DNA sequences spanning the three 21-bp repeats to the binding of CREB or CREB/Tax *in vitro* and gene expression *in vivo* and the possible structural changes imposed on the TRE intervening sequences were studied.

Effect of the TRE repeats on HTLV-1 basal gene expression

The three TREs, almost identical in sequence, did not exhibit differences in the efficiency of binding of CREB to the various TREs in EMSA (not shown) and footprint

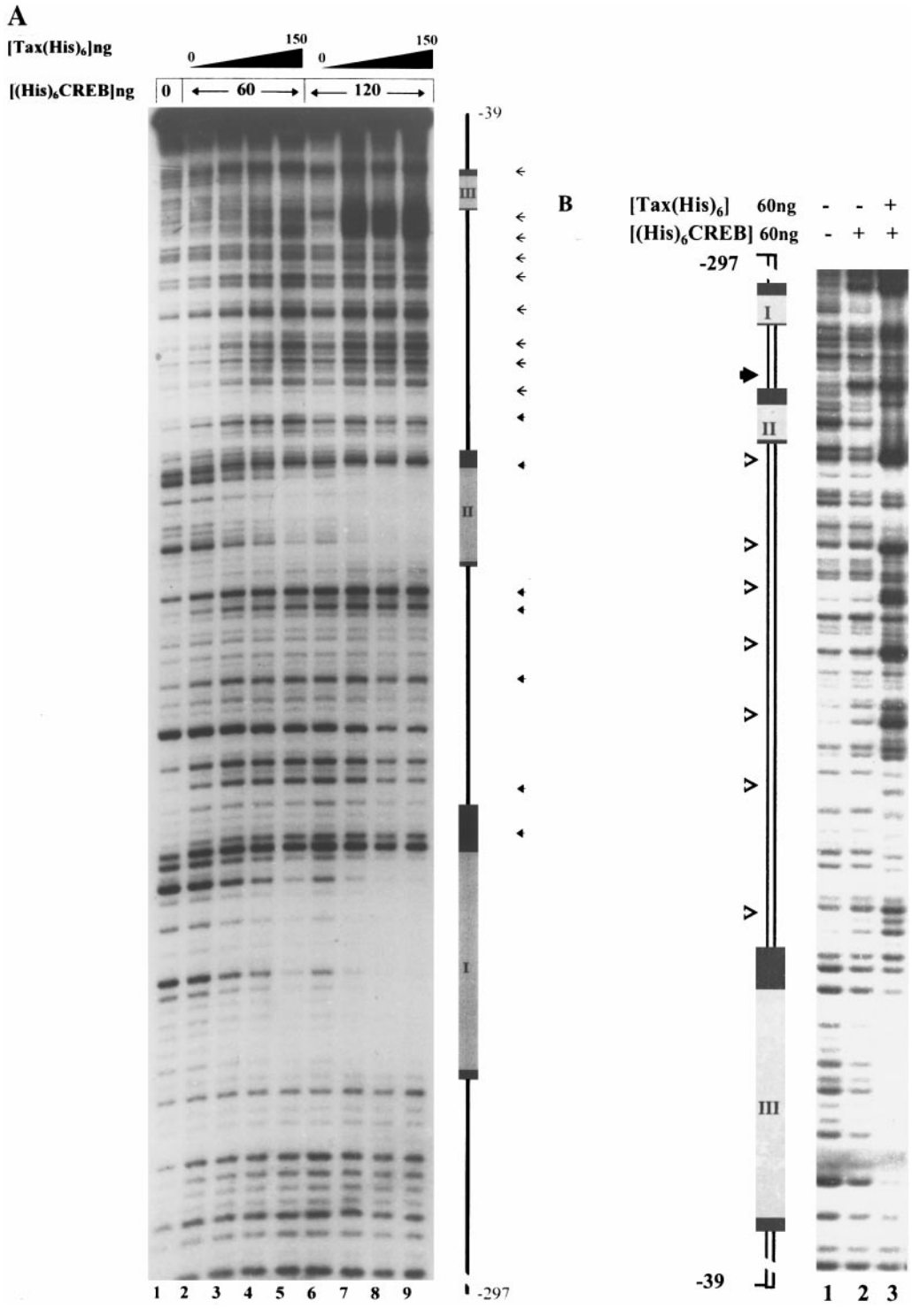

FIG. 5. DNase I analysis of the effect of Tax on CREB binding to the HTLV-1 LTR. (A) The effect of HTLV-1 Tax on CREB binding to the sense strand of the wild-type LTR probe (see Figs. 3 and 4 for details) is shown. Increasing amounts of Tax $(His)_6$ (0, 50, 100, and 150 ng) were incubated with the DNA probe in the presence of 60 ng $(His)_6$CREB (lanes 2–5, respectively) or 120 ng $(His)_6$CREB (lanes 6–9, respectively). The DNA probes were subjected to DNase I digestion as described (Fig. 2). A schematic diagram of the wild-type LTR, including the three TREs, the CREB protected tracts, and the CREB-mediated DNase I hypersensitive bands, is illustrated (see Fig. 2). The Tax-dependent DNase I hypersensitive bands, between TRE II and TRE III, are indicated by arrows, and the CREB-dependent hypersensitive bands are indicated by bold arrowheads. (B) To better illustrate the Tax-dependent DNase I hypersensitive bands between TRE I and TRE II, the effect of Tax on CREB binding to the antisense strand is shown. DNA probes were incubated with CREB (60 ng) in the absence (lane 2) or in the presence (lane 3) of Tax (60 ng, lane 3), prior to DNase I digestion, and the DNA digestion products were analyzed as described above. DNase I digestion of the antisense strand is shown in lane 1.

experiments (Fig. 4). However, each of the triple TRE sites affects the basal gene expression differently (Fig. 1). It is quite clear that TRE II plays a predominant role in CREB-dependent transcription. Mutating TRE II diminishes the LTR-mediated basal expression by close to 90%. The basal expression mediated by TRE II alone in NIH3T3 and Jurkat cells is almost as efficient as that mediated by TRE I and TRE II together (compare U3R13mt to U3R3mt). The contribution of TRE III to the basal expression is greater than that of TRE I, which seems to play only a minor role in modulating basal gene expression (U3R1mt) (see Fig. 1). In general, these results are in agreement with the observation of Barnhart *et al.* (1997). The difference between our work and that of Barnhart *et al.* might result from the different cell lines used by either group (HeLa vs NIH3T3 Jurkat and 293) or from the experimental designs, for example, normalized firefly luciferase activity to Renilla luciferase activity, employing basically the same cell lysis method and the same measuring techniques for both enzyme activities, Barnhart *et al.* (1997) controlled the *cat* gene expression with a *luc* expressing control plasmid.

The different efficiency of gene expression demonstrated by each of the TREs is probably not due to the minor differences in their sequences, but rather to the distances between the *cis* elements. This assumption is supported by several different experiments: (1) The basal activity of a mutant in which TRE II was placed at the same distance from TRE III as TRE I is from TRE III is similar to the activity measured following transfection with the plasmid construct in which TRE II was mutated and the gene expression was mediated by TRE III and I (Fig. 3). (2) Our footprint experiments utilizing increasing concentrations of CREB in the reaction with wild-type HTLV-1 LTR (Fig. 4) did not reveal preferential binding to a particular TRE. (3) Competition footprint experiments, using an increased concentration of competitive DNA, did not reveal preferred protection of a specific TRE (data not shown).

It seems that a minimal distance between two TREs is required for CREB activation of the HTLV-1 promoter. Increasing the distance between TRE III and the upstream TRE (e.g., U3R1mt21d, U3R1mt21o, and U3R2mt, Fig. 3A) hampered the ability of CREB to activate HTLV-1 LTR-dependent gene expression more than the inactivation of either TRE I or TRE III. It is possible that distance-dependent interaction of any two TRE/CREB complexes implementing DNA conformational changes or binding of other transcription factors, such as the TIF or THP proteins between TRE II and TRE III, dictates the efficiency of gene expression mediated by CREB bound to the TREs.

Therefore, it seems that two TREs located at an optimal distance from each other are required for efficient and functional CREB/DNA interactions and the levels of basal gene expression.

Effect of the TREs on Tax transactivation

It was previously demonstrated that Tax markedly stimulates CREB binding to the 21-bp repeats (Anderson and Dynan, 1994; Brauweiler *et al.,* 1995; Yin and Gaynor, 1996; Zhao and Giam, 1992). This binding stabilization by Tax appears to be accomplished by an increase in CREB dimerization and by stabilization of the helical structure of CREB's bZIP domain (Baranger *et al.,* 1995; Perini *et al.,* 1995; Wagner and Green, 1993). It was suggested that Tax forms a ternary complex with CREB bound to CRE in the viral promoter. Using DNase I protection analysis, it was demonstrated that Tax does not change the CREB/TRE DNase I protection pattern (Paca-Uccaralertkun *et al.,* 1994; and our study). However, a more detailed footprint employing the MPE:Fe method demonstrated that Tax enhances the protection of two bases in the G/C-rich region outside of the CRE site. Concomitantly, Tax serves as a high-affinity bridging molecule that directly recruits CBP to the viral promoter in the absence of CREB phosphorylation (Giebler *et al.,* 1997; Kwok *et al.,* 1996). On the basis of the results presented in this work, we propose an expanded model for Tax transactivation of transcription. Our footprint analyses of CREB binding of HTLV-1 LTR indicate clearly that a two- to threefold increase in the efficiency of CREB interaction with the TRE sites occurs in the presence of Tax. This increase in binding is probably reflected in the moderate enhancement of Tax activation of gene expression by HTLV-1 promoters harboring only a mono-TRE (Fig. 1).

Our results clearly indicate that Tax suppresses the prominent reduction in HTLV-I promoter activity caused by the mutation that prevented the binding of CREB to TRE II. The level of gene expression in the TRE II mutated plasmid increases from about 10–20% to about 40–50% in presence of Tax, relative to the wild-type LTR basal gene expression, resulting in a 90- to 130-fold increase of activation. In parallel, an enhancement of DNase I hypersensitive bends is noticed in the spacer sequences between TRE II and TRE III in the presence of Tax (Fig. 5). It is tempting to propose a model by which Tax functions as a bridging molecule that juxtaposes the ternary complexes of TRE I and/or TRE II to TRE III complexes, generating the appropriate structural changes that might be required for the activation of gene expression. It can be summarized that the increased distance between TRE I and TRE III, generated either by the mutating TRE II or by increasing the distance of TRE II from TRE III, resulted in diminished basal gene expression, which can be suppressed by Tax.

The effect of Tax on the activation of the HTLV-1 gene expression can be considered on two levels: first, maximal gene expression, and second, the fold of activation. The highest gene expression is achieved in the presence of the three TREs, and the presence of any two TREs results in a much better HTLV-1 LTR-mediated

gene expression (35–50% relative to wild type) than the presence of a mono-TRE (3–13% relative to wild type). However, it is obvious that TRE III is more essential to Tax activity than the other two TREs, whether as a mono-TRE (U3R12 mt) or in combination with another TRE. This observation can be explained in light of the model proposed recently by Giebler *et al.* (1997), indicating that Tax is part of the bridging between CREB/CBP at TRE III and P/CAF, one of the initiation complex proteins (Giebler *et al.,* 1997). This explanation is also supported by the fact that Tax activation of gene expression by TRE III only is better than that observed for the other two TREs.

Thus, we may summarize that while TRE II plays a crucial role in CREB-mediated activation, TRE III, the proximal TRE relative to the TATA box, is most essential for Tax-mediated activation. Moreover, in addition to the stabilization of the ternary complexes, CREB/Tax/TRE, Tax is probably involved in a cross-talk between the complexes. These changes result in up-regulation of gene expression mediated by the HTLV-1 promoter.

MATERIALS AND METHODS

Expression and purification of recombinant Tax and CREB

Tax $(His)_6$ protein was expressed in *Escherichia coli* HB 101 and purified according to the methods of Zhao and Giam (1991). Human $(His)_6$ CREB was expressed in *E. coli* strain BL-21 (DE3) pLYS (Goren *et al.,* 1995) and purified as follows.

Bacterial culture was harvested and 1 g of bacterial pellet was resuspended in 5 ml of spermidine mix [2.5 mM EDTA, pH 8.0, 200 mM NaCl, 20 mM spermidine, and 14 mM β-mercaptoethanol (β-Me)] and frozen in liquid N_2. One volume of lysis buffer (25 mM Tris pH 8.0, 120 mM NaCl, 3.5% w/v sucrose, 0.2% Brij 58, 1.25 mM EDTA, 10 mM spermidine, 2 mM DTT, and 1 mM PMSF, and 0.5 mg/ml lysozyme) was added, and the bacterial mix was incubated for a further 45 min on ice. The lysate was then incubated for 4 min at 30°C. The suspension was adjusted to 1 M NaCl and centrifuged (18,000 rpm on a Sorval SS34) for 60 min at 4°C. Proteins in the soluble material were precipitated in 25% ammonium sulfate and dissolved in NTA Ni native column buffer (Goren *et al.,* 1995). Purification of the histidine-tagged CREB was further carried out as previously described (Goren *et al.,* 1995).

Plasmid and probe construction

The plasmid pU3RI (Sodroski *et al.,* 1984) was digested with *Bgl*II and cohesive ends were treated with mung bean nuclease followed by *Xho*I digestion. A 0.75-kb DNA fragment containing the U3, R, and 105 bp from the U5 region of the HTLV-1 LTR was excised and cloned into pALTER-1 (Promega) via the *Hin*dIII-filled-in and *Sal*I restriction sites, generating plasmid pALTERU3R. This plasmid served for the *in vitro* site-directed mutagenesis of the HTLV-1 LTR (described below). For *in vivo* luciferase assay, the viral promoter sequences (both wild-type and mutants) were excised from the various pALTERU3R plasmids by *Bam*HI and *Hin*dIII digestion and fused to the firefly luciferase or the Renilla luciferase reporter genes of pGL3 or pRL-null, respectively (Promega), to give rise to the various pGLU3R and pRLU3R expression vectors.

Site-directed mutagenesis

Site-directed mutagenesis was carried out using the Alter II mutagenesis kit (Promega) according to the manufacturer's instructions. The oligonucleotides (5′) CCAGACTAAGGCTCACTAGTCTCCCCCCGGAGG, (5′) CGGCTCGGGCTAGATCTTAACGTTTCCCCCTGAAG, and (5′) CCGTCCTCAGGCCTCGAGGACAACCCCTCAC, corresponding to the first, second, and third TRE sites on the HTLV-1 LTR, respectively, were used to generate single- or double-point mutations on the viral LTR. The specificity of the mutation was ensured by sequencing of the mutated promoters. To generate an *Eco*RI mutation at position −180 (Seiki *et al.,* 1982), oligonucleotide (5′) GTGTCCCCCTGAATTCAAATCATAAGC was used.

DNase I footprinting assay

DNA fragments for DNase I protection experiments were prepared by PCR, using 5′ end-labeled DNA oligonucleotides, corresponding to the sites of choice on the HTLV-1 LTR and purified following a 3.0% agarose gel (NuSieve) electrophoresis.

For the DNase I footprinting experiments, promoter fragments (40,000 to 60,000 cpm) were incubated for 25 min at room temperature in the absence or in the presence of various amounts of $(His)_6$CREB, or $(His)_6$CREB and Tax$(His)_6$ in 100 μl of binding buffer (10 mM Tris, pH 7.6; 50 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM spermidine, 2 mM DTT, 25 μM $ZnCl_2$, 10% glycerol, and 0.1 μg/μl BSA). RQ DNase I (0.25 units; Promega) was added directly to the binding reaction. Nucleolytic digestion was stopped after 2.5 min by addition of 100 μl of stop buffer (200 mM NaCl, 30 mM EDTA, 0.2% SDS, and 200 mg/μl yeast tRNA). The DNA was phenol-extracted and ethanol-precipitated. One-third of the footprint reaction was analyzed on sequencing gel in parallel to DNA sequencing reaction of the corresponding DNA fragment using the *fmol* DNA sequencing system (Promega).

Transfection

NIH 3T3 and 293 cell lines were utilized for transient transfection using the standard calcium phosphate methods. Cells were plated 24 h prior to transfection in Dulbecco's modified Eagle's medium containing 10% fetal calf serum to give a semiconfluent culture. In all experiments the ratio of the Tax producing plasmids pCTax to reporter

plasmid DNA (0.5 μg) was 1:5. The ratio of the various HTLV-1 LTR wild-type or mutant HTLV-1 LTR–firefly transcription fusion reporter vectors to the transfection control, pRLU3R harboring the HTLV-1 LTR–Renilla transcription fusion DNA fragment, was 4:1. The culture medium was changed 24 h posttransfection, and 24 h later the cells were harvested into passive lysis buffer (Promega) and the amount of light emission was determined using an automatic Anthos I Lucy photoluminometer.

Jurkat cells were transfected using Transfast reagent (Promega). In all experiments the ratio of the reporter plasmids (1 μg) to the Tax-producing plasmid, pcTax DNA, was 2.5:1, and the ratio to the pRL-CMV (Promega) DNA was 200:1. Twenty-four hours before transfection, Jurkat cells were diluted to 0.8×10^6 cells/ml in RPMI medium containing 10% FCS. On the day of transfection, 1×10^6 cells for each sample were pelleted and resuspended in 1 ml RPMI containing the DNA and Transfast reagent at a molar ratio of 1:1. Following incubation at 37°C for 1 h, 1 ml of RPMI containing 20% FCS was added. The cells were harvested 48 h later.

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